

Human and *Helicobacter pylori* Interactions Determine the Outcome of Gastric Diseases

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Abstract The innate immune response is a critical hallmark of *Helicobacter pylori* infection. Epithelial and myeloid cells produce effectors, including the chemokine CXCL8, reactive oxygen species (ROS), and nitric oxide (NO), in response to bacterial components. Mechanistic and epidemiologic studies have emphasized that dysregulated and persistent release of these products leads to the development of chronic inflammation and to the molecular and cellular events related to carcinogenesis. Moreover, investigations in *H. pylori*-infected patients about polymorphisms of the genes encoding CXCL8 and inducible NO synthase, and epigenetic control of the ROS-producing enzyme spermine oxidase, have further proven that overproduction of these molecules impacts the severity of gastric diseases. Lastly, the critical effect of the crosstalk between the human host and the infecting bacterium in determining the severity of *H. pylori*-related diseases has been supported by phylogenetic analysis of the human population and their *H. pylori* isolates in geographic areas with varying clinical and pathologic outcomes of the infection.

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1 *Helicobacter pylori*: From Infection to Diseases

Helicobacter pylori is a Gram-negative microaerophilic bacterium that specifically colonizes the human stomach and exhibits extraordinary genetic diversity. Long-term infection causes diseases including chronic active gastritis, peptic ulcers, B cell lymphoma of mucosa-associated lymphoid tissue, and adenocarcinoma. The bacterium is adapted to its hostile ecologic niche through the activity of its bacterial urease that neutralizes gastric acidity by generating ammonium from urea. Moreover, the common trait of *H. pylori* strains that have increased risk of inducing gastric adenocarcinoma is the expression of specific virulence genes. First, the cytotoxin-associated gene A (CagA) is a bacterial factor that belongs to the *cag* pathogenicity island (*cagPAI*) and is injected in human epithelial cells through a type IV secretion system (T4SS). CagA is then sequentially tyrosine-phosphorylated by the host SRC proto-oncogene, non-receptor tyrosine kinase (SRC, c-Src) and ABL proto-oncogene 1, non-receptor tyrosine kinase (ABL1) kinases (Mueller et al. 2012) and dysregulates the homeostatic signal transduction of gastric epithelial cells (Higashi et al. 2002). This results in a persistent inflammation through the induction of CXCL8 (also known as IL-8) synthesis, and malignancy by loss of cell polarity, resistance to apoptosis, and chromosomal instability (Umeda et al. 2009). Second, the vacuolating cytotoxin A (VacA) contributes to *H. pylori* pathogenesis by regulating the inflammatory process (Supajatura et al. 2002) and by damping cell death by autophagy, thus favoring gastric colonization and oxidative damage (Raju et al. 2012). Although the contribution of VacA to gastric metaplasia has not been directly demonstrated using animal models, epidemiological studies have emphasized a correlation between the

vacA gene structure and severity of *H. pylori*-related diseases; the signal region s1 and the middle region m1 of the *vacA* gene are associated with strains at increased risk for inducing peptic ulcers and/or gastric cancer, compared to s2 or m2 strains (Atherton et al. 1997).

However, the sole expression of virulence factors is not sufficient to explain *H. pylori* pathogenesis and the development of gastric cancer. Environmental components, including iron deficiency (Noto et al. 2013) or high-salt diet (Fox et al. 2003), have been implicated in the outcome of *H. pylori* infection. Moreover, the involvement of host factors in gastric cancer risk is reflected in polymorphisms in genes that govern inflammation, stomach homeostasis (Vinall et al. 2002), and apoptosis/proliferation (Menheniott et al. 2010), as well as epigenetic factors resulting in altered levels of DNA methylation of specific genes (Schneider et al. 2015).

The backbone of *H. pylori*-associated inflammation is the non-specific activation of gastric epithelial cells and the presence of polymorphonuclear neutrophils (PMN), antigen presenting cells, i.e., dendritic cells and macrophages, and the adaptive immune response associated with CD4+ cells of the Th1 and Th17 subtypes in the gastric mucosa (Wilson and Crabtree 2007). The ultimate goal of the innate immune response of stromal and myeloid cells in response to *H. pylori* is to limit colonization by the pathogen; this occurs either indirectly by recruiting immune cells through the synthesis of chemokines, such as CXCL8, or directly by releasing antimicrobial effectors including reactive oxygen species (ROS) and nitric oxide (NO). However, these inducible host factors may also have deleterious effects by favoring persistent inflammation, which can be critical for the progression of gastric diseases according to the Correa cascade model (Correa 1988), and/or by affecting carcinogenesis through the regulation of cellular events (apoptosis/proliferation) or the induction of genetic changes (oxidative damage; mutations).

Here, we review the induction and the role of three major mediators of the innate immune response, namely the chemokine CXCL8, ROS, and NO during *H. pylori* infection.

2 The Chemokine CXCL8

The production of chemokines, and notably CXCL8, by gastric epithelial cells is a recurrent feature of *H. pylori* infection and represents a striking example of how the crosstalk between the host and the bacterium may affect the severity of *H. pylori*-associated diseases. Indeed, CXCL8 is a member of the CXC chemokine family produced by the innate response of myeloid and epithelial cells and is a major mediator of inflammation, acting as a chemoattractant for neutrophils and T cells (Fig. 1). Moreover, CXCL8 acts as a potent angiogenic factor in endothelial cells by stimulating vascular endothelial growth factor expression and enhancing cell

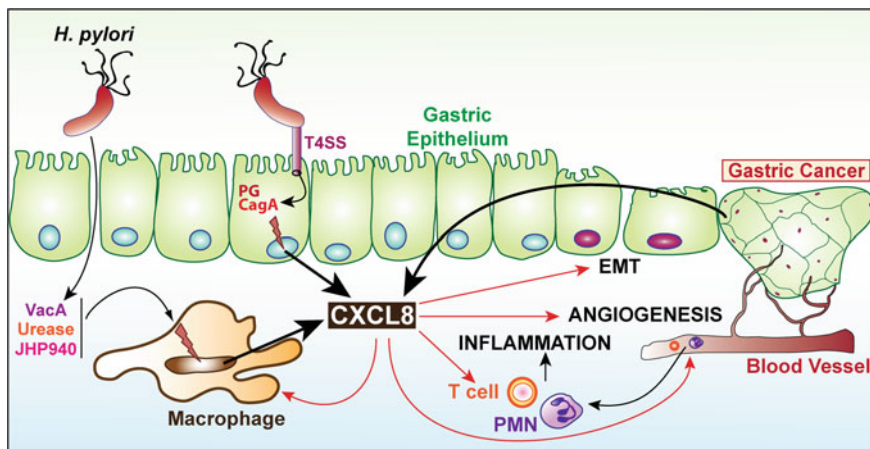


Fig. 1 Hypothetical model for CXCL8-mediated gastric carcinogenesis. Gastric epithelial cells and macrophages produce CXCL8 (also referred to as IL-8) in response to different *H. pylori* factors; VacA, urease, and JHP940 have been shown to stimulate macrophages, whereas the T4SS-dependent injection of CagA or peptidoglycan (PG) has been involved in epithelial cell activation. CXCL8 chemoattracts PMNs and T cells to infiltrate to the site of infection and activates immune cells, thus favoring a persistent inflammatory state. Moreover, CXCL8 stimulates epithelial-mesenchymal transition (EMT) and angiogenesis. These events may contribute to *H. pylori* carcinogenesis. Lastly, tumor cells also produce CXCL8, further potentiating gastric cancer development

proliferation and survival, potentiating epithelial-mesenchymal transition, and activating immune cells at the tumor site (Yuan et al. 2005) (Fig. 1).

The expression of *CXCL8* mRNA and the production of CXCL8 by antral biopsies are increased in *H. pylori*-infected patients compared to *H. pylori*-negative cases, and interestingly, CXCL8 has been immunodetected in both epithelial cells and macrophages (Ando et al. 1996). Further, increased CXCL8 in gastric biopsies is associated with infection by *cagA*-positive *H. pylori* (Li et al. 2001), and the serum concentration of CXCL8 in *H. pylori*-positive patients with gastric cancer is more elevated than those without carcinoma (Konturek et al. 2002) (Fig. 1). Consequently, it has been described that CXCL8 is a critical factor for the development, severity, and spread of gastric carcinoma (Kitadai et al. 1999; Lee et al. 2004; Asfaha et al. 2013).

2.1 Induction of *CXCL8* by *H. pylori*

Numerous investigations have analyzed the mechanisms by which *H. pylori* interacts with epithelial cells to stimulate CXCL8 production. Studies have highlighted the critical role of the T4SS in this process (Fischer et al. 2001; Belogolova

et al. 2013; Varga et al. 2016) (Fig. 1). Although several bacterial components, such as peptidoglycan (Viala et al. 2004) or DNA (Varga et al. 2016), can be translocated into host cells through the T4SS and induce an innate immune response, the role of CagA has been evidenced by the following discoveries: (i) *H. pylori* *cagA*-positive strains induce more CXCL8 than *cagA*-negative strains (Crabtree et al. 1994; Salih et al. 2014); (ii) *cagA* mutant strains have reduced ability to stimulate CXCL8 (Brandt et al. 2005; Gobert et al. 2013); (iii) ectopic expression of CagA in gastric epithelial cells stimulates CXCL8 expression (Kim et al. 2006); and (iv) inhibition of CagA phosphorylation decreases CXCL8 mRNA expression (Gobert et al. 2013). Nevertheless, the effect of CagA on CXCL8 induction has not been observed in some studies (Sharma et al. 1995), and the various results could be explained by differences in the type of host cells, in the *H. pylori* strains that have been used, and in the time of infection. Remarkably, it has been reported that *H. pylori*-stimulated CXCL8 production by AGS cells requires phosphorylation of CagA in the early stage of the infection, and then both phospho-CagA and a conserved motif, termed conserved repeat responsible for phosphorylation-independent activity (CRPIA), are implicated in late cell activation (Suzuki et al. 2009).

Again, according to the design of the in vitro experiments, different results have been observed regarding the signal transduction leading to *H. pylori*-induced CXCL8 induction in epithelial cells. Either the pro-inflammatory pathway involving the mitogen-activated protein kinase (MAPK) p38 and the transcription factor nuclear factor-kappa B (NF- κ B) (Yamaoka et al. 2004; Gobert et al. 2013) or the extracellular signal-regulated kinases 1/2 (ERK1/2, MAPK1/3)-related signals (Keates et al. 1999; Brandt et al. 2005) have been described to play a role in inducible CXCL8 transcription. Of note, p38 and NF- κ B activation by *H. pylori* is partially mediated by CagA (Allison et al. 2009; Lamb et al. 2009; Gobert et al. 2013), but other *H. pylori* factors, including the outer inflammatory protein OipA (Lu et al. 2005a) and peptidoglycan (Allison et al. 2009), are possibly involved in the stimulation of p38 phosphorylation/activation. Finally, it is evident that multiple ways of signaling can lead to CXCL8 induction in *H. pylori*-infected epithelial cells, as underlined by work showing that the transcription factors activator protein-1 (AP-1) and NF- κ B are required for maximal induction of CXCL8 mRNA expression (Chu et al. 2003).

Myeloid cells are also a major source of CXCL8 during pathological conditions (Fig. 1). Thus, RNA in situ hybridization has revealed that CXCL8 mRNA is expressed in macrophages and neutrophils in the gastric lamina propria of patients with chronic active *H. pylori* gastritis (Eck et al. 2000). Human peripheral blood mononuclear leukocytes or the human monocyte cell lines THP-1 or U937 stimulated in vitro with *H. pylori* urease (Harris et al. 1996), an *H. pylori* water extract (Bhattacharyya et al. 2002), the *H. pylori* protein JHP940 (Rizwan et al. 2008), or VacA (Hisatsune et al. 2008) express CXCL8 mRNA and produce CXCL8. Moreover, *H. pylori*-induced CXCL8 production may occur independently of the T4SS because a *cagE* mutant was shown to retain its inducing activity (Maeda et al.

2001). CXCL8 induction in response to *H. pylori* is inhibited in THP-1 cells by ERK1/2, p38, and NF- κ B inhibitors, demonstrating the involvement of the MAPK/NF- κ B pathway in this process. However, VacA directly increases CXCL8 production in pro-monocytic U937 cells by activation of the p38 MAPK leading to the activation of two transcription factors, namely the heterodimer activating transcription factor 2/c-AMP response element-binding protein-1 (CREB1) that binds to the AP-1 site of the CXCL8 promoter and NF- κ B (Hisatsune et al. 2008).

2.2 CXCL8 Gene Polymorphisms

The CXCL8–251 A/A genotype is associated with a higher risk of atrophic gastritis and gastric cancer compared with the A/T or T/T genotype in Japanese (Ohyauchi et al. 2005; Taguchi et al. 2005), Chinese (Lu et al. 2005b; Zhang et al. 2010), and Korean (Kang et al. 2009) populations. Importantly, the CXCL8 promoter activity is enhanced, and CXCL8 is produced in greater amounts in the gastric mucosa of *H. pylori*-infected Asian patients harboring the A/A genotype than those with the A/T or T/T alleles (Ohyauchi et al. 2005; Taguchi et al. 2005; Ye et al. 2009; Song et al. 2010), demonstrating a direct link between the gene polymorphism and phenotypic identity. In the same way, it has been evidenced that the CXCL8–251 A allele is correlated with the degree of neutrophil infiltration, atrophy, and intestinal metaplasia (Taguchi et al. 2005; Ye et al. 2009). Interestingly, it has been reported that in Korean patients, the IL-10–592 A/A genotype (low promoter activity of the IL-10 gene) and the CXCL8–251 A/A genotype (high promoter activity) each are associated with a greater relative risk of developing gastric adenocarcinoma, and the combination yielded a synergistic increase in risk (Kang et al. 2009). Such findings suggest that a combination of multiple host immune factors is involved in carcinogenesis.

Inversely, the CXCL8–251T/T genotype is associated with gastric carcinoma exhibiting a high frequency of microsatellite instability, antral location, and greater depth of invasion in Japanese patients (Shirai et al. 2006) and with an increased risk of adenocarcinomas in a population of Chinese Veterans infected with *H. pylori* (Lee et al. 2005). It should be noted that in the latter study, the T allele is correlated with increased transcriptional activity in comparison with the –251A counterpart (Lee et al. 2005), which is in contrast with previous findings (Ohyauchi et al. 2005; Taguchi et al. 2005; Ye et al. 2009; Song et al. 2010). Similarly, the frequency of the A/A genotype at the position –251 in the CXCL8 promoter in European Caucasian patients is significantly increased in patients with gastritis, but it is not correlated with gastric cancer (Szoke et al. 2008). Interestingly, a study performed in Brazil showed that the CXCL8–845 T/C polymorphism, but not the CXCL8–251 A/T, is correlated with increased risk of developing chronic gastritis and gastric cancer in *H. pylori*-infected individuals (de Oliveira et al. 2015). The authors also show that the C variant in position –845 is responsible for the presence of the

binding sites for the transcription factors NF- κ B and CREB1 (de Oliveira et al. 2015), which are involved in increased *CXCL8* gene expression. Of importance, no association between the *CXCL8*-251A/T polymorphism and the risk of *H. pylori* infection has been found (Lee et al. 2005), demonstrating that the modulation of chemokine production does not affect the level of *H. pylori* infection.

Together, these data indicate that *CXCL8* plays a major function in *H. pylori* pathogenesis. Although the *IL8*-251A/T polymorphism might be a relevant host susceptibility factor for development of gastric cancer, this association is likely to be ethnicity-specific, as previously suggested by a meta-analysis (Canedo et al. 2008). More studies relating human genetic backgrounds, specific gene polymorphisms, and disease outcomes are needed.

3 Induction and Role of ROS

The ultimate goal of the inducible generation of ROS by stromal or myeloid cells in response to pathogenic bacteria is to limit the development of intruders. Nonetheless, persistent production of ROS affects cell signaling and DNA damage that may ultimately lead to carcinogenesis. Two main biochemical pathways leading to ROS production, namely NADPH oxidase and spermine oxidase (SMOX), have been identified in *H. pylori* infection. The mammalian NADPH oxidases are enzymes sharing the capacity to transport electrons across the plasma membrane and to generate superoxide. Seven homologs have been identified (NOX1, NOX2/gp91^{phox}, NOX3, NOX4, NOX5, DUOX1, and DUOX2), but NOX1 and NOX2 are of particular interest for *H. pylori* pathogenesis because NOX1 is highly expressed in the gastrointestinal tract and NOX2, the prototype NADPH oxidase, is abundant in immune cells (Fig. 2). SMOX is expressed in epithelial cells and macrophages and synthesizes hydrogen peroxide through the oxidation of the biogenic polyamine, spermine (Fig. 2).

3.1 ROS During *H. pylori* Infection

Several lines of evidence suggest that *H. pylori* infection is associated with increased ROS production in the stomach. A chemiluminescent method has been used to demonstrate that ROS generation is increased in gastric biopsies of *H. pylori*-positive patients compared to those from uninfected subjects (Papa et al. 2002). Moreover, H₂O₂ generation, assessed by an electrochemical microsensor positioned close to the gastric mucosa, was shown to be increased in Mongolian gerbils infected with *H. pylori* SS1 (Elfvin et al. 2007).

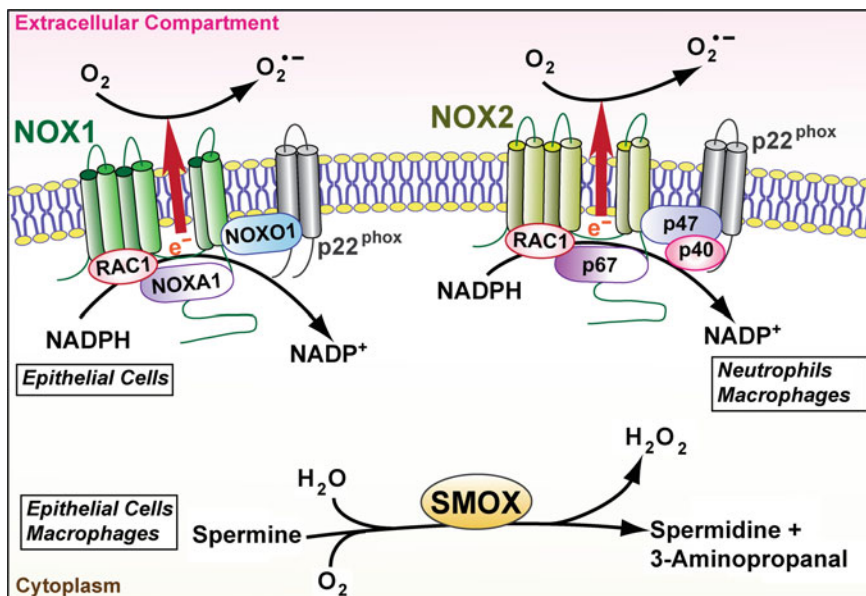


Fig. 2 Structure, function, and cellular localization of the two NADPH complexes, NOX1 and NOX2, and SMOX. The type of gastric cells in which these enzymes have been detected during *H. pylori* infection is indicated in the boxes

A significant correlation between the production of ROS in the gastric tissues of *H. pylori*-infected dyspeptic patients and the level of 8-hydroxy-2-deoxyguanosine (8-OHdG), a biomarker of DNA oxidative damage, has been evidenced in European patients (Papa et al. 2002). However, this was not observed in uninfected individuals and in the few patients infected with *cagA*-negative *H. pylori* (Papa et al. 2002). Of note, Pignatelli et al. (2001) questioned whether eradication of *H. pylori* could be associated with reduced oxidative damage to gastric tissues. They showed by immunohistochemistry in gastritis tissues of infected subjects that NOS2 was primarily detected in inflammatory cells, while nitrotyrosine and 8-OHdG localized mainly to foveolar epithelial cells (Pignatelli et al. 2001). While NOS2 and nitrotyrosine levels decreased with *H. pylori* eradication, the authors did not find a significant reduction of 8-OHdG levels (Pignatelli et al. 2001).

3.2 Expression and Role of NOX1

Although NOX1 expression has never been documented in the normal human stomach (Salles et al. 2005), in vivo and in vitro data suggest that *H. pylori* can induce this enzyme. It has been shown that the genes encoding NOX1 and one of its partner proteins, NOXO1, are upregulated in tissues from *H. pylori*-infected

patients with intestinal-type or diffuse-type gastric adenocarcinomas and were absent in normal stomachs (Tominaga et al. 2007). These authors also showed by immunofluorescence and confocal microscopy that the proteins NOX1 and the partners NOXA1, NADPH oxidase activator 1 (NOXA1), and p22^{phox} co-localize with the Golgi apparatus in gastric cancer cells, and that their expression levels are increased in gastric cancers compared to the surrounding tissue and compared to areas of chronic atrophic gastritis or adenomas in patients without carcinoma (Tominaga et al. 2007). This provides a rationale for the use of NOX1 as a marker of neoplastic transformation and for the role of NOX-1-derived ROS in *H. pylori*-mediated carcinogenesis in the human stomach. In the same way, non-transformed antral-derived primary epithelial cells and the gastric epithelial cell line AGS infected with *H. pylori* 26695 express NOX1 and produce ROS rapidly (within 30 min) and transiently (den Hartog et al. 2016). The transcription factor(s) involved in *H. pylori*-mediated *NOX1* transcription in gastric epithelial cells have not been characterized yet, but one study implicated the pleiotropic signaling molecule Ras-related C3 botulinum toxin substrate 1 (Rac1) (den Hartog et al. 2016). Further work is warranted to delineate the molecular mechanisms leading to NOX1 expression, since these findings could provide critical insight into *H. pylori*-associated inflammation.

Several articles have analyzed the effect of NOX1-derived ROS on cellular and molecular events that may explain *H. pylori* pathogenesis. Ngo et al. (2016) have demonstrated that the oxidative burst induced by *H. pylori* in human gastric epithelial cells is responsible for the phosphoinositide 3-kinase (PI3-K)/AKT serine/threonine kinase 1 (AKT1)/glycogen synthase kinase 3 beta (GSK3 β)-dependent expression of the transcription factor Snail (SNAI1) (Ngo et al. 2016), which is essential for epithelial–mesenchymal transition and therefore in gastric cancer progression and metastasis. Additionally, it has been shown that the transcription factor signal transducer and activator of transcription 3 (STAT3), which plays a major function in inflammation and angiogenesis, is also induced in gastric epithelial cells via upregulated autocrine IL-6 signaling, partially mediated by endogenous ROS (Piao et al. 2016). Lastly, α -lipoic acid, a naturally occurring thiol compound that exhibits antioxidant properties, inhibits NADPH oxidase-derived ROS production in AGS cells and concomitantly dampens NF- κ B and AP-1 activation and expression of the oncogenes β -catenin (CTNNB1) and *c-Myc* (MYC) (Byun et al. 2014). Interestingly, the endonuclease apurinic/aprimidinic endodeoxyribonuclease 1 (APEX1, also known as APE1), a multifunctional enzyme that plays a central role in the cellular response to oxidative stress, including DNA repair and redox regulation of transcriptional factors, is induced by endogenous ROS in AGS cells (den Hartog et al. 2016). APEX1 inhibits RAC1 activation and consequently NOX1-dependent ROS production, and blocking APEX1 leads to sustained NOX1 activation and increased O₂⁻ generation (den Hartog et al. 2016). This could represent a mechanism by which cells limit NOX1-dependent DNA damage and/or a strategy developed by the bacterium, to promote its own survival by inhibiting ROS production.

3.3 Expression and Role of NOX2

Myeloid cells are also a major source of ROS during pathological process. Live *H. pylori*, a bacterial sonicate, or *H. pylori* lipopolysaccharide (LPS) can each induce a rapid oxidative burst in human primary blood-derived polymorphonuclear cells (PMNs) (Nielsen and Andersen 1992; Nielsen et al. 1994; Allen et al. 2005). However, this effect was not observed in neutrophils primed with a water extract of *H. pylori* (Shimoyama et al. 2003). In studies using a Clark oxygen electrode, it has been demonstrated that *H. pylori* triggers a faster and stronger oxidative burst in PMNs than that induced by other bacteria, including *Staphylococcus aureus* and *Salmonella*, or by phorbol myristate acetate (Allen et al. 2005). Of particular interest, *H. pylori* does not efficiently recruit the NADPH oxidase domain p47^{phox} or p67^{phox} to the phagosome; consequently, NADPH oxidase accumulate in patches at the cell surface and the infection leads to the release of large amounts of superoxide into the extracellular milieu (Allen et al. 2005). This unique ability to prevent the oxidase assembly at the phagosome allows *H. pylori* to evade the oxidative killing and may result in neutrophil-derived oxidative damage to the surrounding cells, mainly from the epithelium.

The gene *napA* of *H. pylori* (Evans et al. 1995) encodes the 17 kDa virulence factor neutrophil-activating protein A (NapA) that forms hexagonal rings, binds up to 40 atoms of iron per monomer, and oligomerizes into dodecamers with a central hole capable of binding up to 500 iron atoms per oligomer (Tonello et al. 1999). It has been shown that NapA is a chemoattractant for leukocytes and induces NADPH oxidase in neutrophils through a SRC/PI3K signaling pathway (Satin et al. 2000). In addition to its effect on ROS production by neutrophils, NapA also displays a role in the bacterium itself. Thus, the *napA* mutant is more sensitive to oxygen (Olczak et al. 2002) and oxidative stress induced by organic peroxides, cumene hydroperoxide, or paraquat (Olczak et al. 2002; Wang et al. 2006), than the wild-type strain and exhibits more bacterial DNA damage (Wang et al. 2006). However, no alteration in gastric colonization was observed in animals infected for 3 weeks with the *napA* mutant compared to the wild-type strain (Wang et al. 2006). Chronic infection of mice or the use of animal models of *H. pylori*-mediated gastric dysplasia, e.g., transgenic FVB/N insulin-gastrin (INS-GAS) mice that over-express pancreatic gastrin or gerbils, would be useful to determine the role of NapA and the associated induction of NOX2 in carcinogenesis.

Similarly, the stimulation of blood-derived monocytes or differentiated THP-1 cells with *H. pylori* or its LPS yields a rapid and sustained production of O₂⁻ (Mai et al. 1991). More particularly, it has been reported that *H. pylori* cecropin-like peptide Hp(2-20) is a chemoattractant and activator for monocytes through the N-formyl peptide receptors 1 and 2, and that monocytes stimulated with Hp(2-20) release ROS, which inhibit the function of antineoplastic lymphocytes (Betten et al. 2001).

Chronic granulomatous disease (CGD) mice are animals with a targeted disruption of the NOX2/gp91^{phox} subunit of the NADPH oxidase. At 12- and 30-week post-infection with *H. pylori*, CGD mice showed no differences in colonization, but more glandular atrophy, proliferation of gastric epithelial cells, and neutrophil infiltration (Keenan et al. 2005). But overall, there were no significant changes in gastritis score between wild-type and CGD mice (Keenan et al. 2005). In contrast, a second study reported that gp91^{phox-/-} mice exhibit more inflammation and increased mononuclear cell infiltration in the mucosa during infection with *H. felis* or *H. pylori* (Blanchard et al. 2003). Although this investigation was performed at 3-weeks post-infection, which is not sufficient time to observe strong gastric inflammation, the results are consistent with in vitro data showing that the partial scavenging of ROS by *N*-acetylcysteine led to a decrease of *H. pylori*-induced inflammasome formation, and IL-1 β and IL-18 production by *H. pylori*-infected THP-1 cells (Li et al. 2015).

The NOX1 and NOX2 partner p22^{phox} is essential for NADPH oxidase activity. It has been reported that the C242T polymorphism of the p22^{phox} gene, which leads to reduced production of ROS, is associated with reduced risk of developing functional dyspepsia and intestinal metaplasia in *H. pylori*-infected patients (Tahara et al. 2009a, b).

In summary, NADPH-derived ROS may be both a consequence of and contributor to the severity of *H. pylori* gastritis and may contribute to the progression to precancerous lesions. However, additional studies are needed to further assess the role of this source of ROS in carcinogenesis.

3.4 Expression and Role of SMOX

An important characteristic of the innate immune response of the host during *H. pylori* infection is the induction of the enzyme arginase 2, but not arginase 1, through an NF- κ B-dependent pathway (Gobert et al. 2002). Arginases are enzymes that catabolize L-arginine into urea and L-ornithine; this last product is then converted by ornithine decarboxylase (ODC) into the first polyamine putrescine, which is then sequentially catabolized to spermidine and spermine. Although ODC is mainly regulated at the post-transcriptional level in many cell types, *Odc* mRNA expression, and consequently, ODC protein is increased in murine macrophages infected in vitro with *H. pylori* (Gobert et al. 2002) and in gastric macrophages of infected mice and humans (Chaturvedi et al. 2010), and this favors the synthesis of the three polyamines (Gobert et al. 2002; Chaturvedi et al. 2010). Importantly, the back-conversion of spermine to spermidine occurs through the enzyme SMOX, which oxidizes spermine into spermidine, 3-aminopropanal, and H₂O₂ (Wang et al. 2001). We have reported that SMOX is induced at the transcriptional level by *H. pylori* in macrophages (Chaturvedi et al. 2004) and in gastric epithelial cells (Xu et al. 2004), thus favoring apoptosis and DNA damage by an H₂O₂-dependent mechanism (Chaturvedi et al. 2004; Xu et al. 2004).

Two *cagA*-deficient strains of *H. pylori* and a *cagE* mutant each induced less *SMOX* mRNA expression in conditionally immortalized murine gastric epithelial cells and in human epithelial cells than the wild-type strains (Chaturvedi et al. 2011), suggesting that CagA and the T4SS are involved in *SMOX* expression. These in vitro observations were confirmed by the analysis of gastric tissues from patients with *H. pylori* infection: individuals infected with *cagA*-positive *H. pylori* showed a significant increase of *SMOX* mRNA expression, and *SMOX* protein level in the gastric epithelium compared to patients harboring *cagA*-negative strains (Chaturvedi et al. 2011). Similar data were obtained in mice infected with *cagA*-positive *H. pylori* versus a *cagE* mutant, and in gerbils infected with *cagA*-positive versus *cagA*-negative strains (Chaturvedi et al. 2011, 2015).

SMOX expression in isolated epithelial cells from the gastric tissues of *H. pylori*-infected gerbils correlates with 8-oxoguanosine formation (Chaturvedi et al. 2011); there is a subpopulation of *SMOX*-expressing cells with oxidative DNA damage that were resistant to apoptosis (Chaturvedi et al. 2011), thus increasing the likelihood to favor carcinogenesis. In vitro, inhibition of *SMOX* with MDL 72527 (N^1, N^4 -Di(buta-2,3-dien-1-yl)butane-1,4-diamine dihydrochloride) reduced *H. pylori*-induced 8-oxoguanosine levels, emphasizing the critical role of this enzyme in carcinogenesis. This was further evidenced by in vivo experiments: treatment with either α -difluoromethylornithine, an inhibitor of ODC, or MDL 72527, reduced gastric dysplasia and carcinoma in *H. pylori*-infected gerbils (Chaturvedi et al. 2015). Moreover, the subpopulation of 8-oxoguanosine+ cells that were resistant to apoptosis (8-oxoguanosine^{high}, active caspase-3^{low}) has been significantly reduced in the gastric mucosa of infected animals treated with the ODC or *SMOX* inhibitor (Chaturvedi et al. 2015).

Excitingly, reduced levels of *SMOX*, DNA damage, and DNA damage^{high}, apoptosis^{low} cells has been also observed in gastric epithelial cells grown from mice lacking the gene encoding epidermal growth factor receptor (EGFR) or in mice with disruption of EGFR signaling (*Egfr*^{wa5} mice) (Chaturvedi et al. 2014), implicating this receptor in *H. pylori*-mediated *SMOX* expression. Additionally, the kinase erb-b2 receptor tyrosine kinase 2 (ERBB2) was shown to be critical for the cellular events associated with EGFR signaling in gastric epithelial cells (Chaturvedi et al. 2014). Further identification of the molecular events leading to *SMOX* transcription is warranted to further understand mechanisms of *H. pylori*-associated carcinogenesis.

3.5 Epigenetic Regulation of *SMOX*

Recently, the epigenetic control of *SMOX* has been evidenced. It has been described that overexpression of the microRNA miR-124 blocks *SMOX* mRNA expression, *SMOX* protein induction, and *SMOX* activity in *H. pylori*-infected AGS cells by targeting the 3'-UTR of the human *SMOX* gene (Murray-Stewart et al. 2016). The promoter region of the tumor suppressor *miR-124* is hypermethylated in

a variety of cancers, including gastric cancer (Ando et al. 2009). Consistent with this, it was demonstrated that increased methylation of the three *miR-124* loci is associated with increased SMOX protein expression in patients with *H. pylori* infection (Murray-Stewart et al. 2016). Together, the data suggest that in non-transformed cells, *miR-124* is normally expressed and downregulates SMOX, whereas SMOX induction should be maximal during carcinogenesis.

4 Nitrosative Stress

The non-specific defense program of gastric epithelial cells and macrophages against *H. pylori* leads to the production of NO, a ubiquitous free radical synthesized by the enzyme inducible NO synthase 2 (NOS2) through the 5-electron oxidation of the terminal guanidino-N2 of the amino acid L-arginine in the biological milieu. Since the discovery of the immunological properties of the macrophage-derived L-arginine-NO pathway, notably by the work of J.B. Hibbs, Jr in the 1990s (Lancaster and Hibbs 1990), more than 23,000 referenced publications have addressed the thematic field of NOS expression during the immune response and the role of NO in the pathophysiological process of infectious diseases.

4.1 *H. pylori* Infection and NOS2

An increase of *NOS2* mRNA expression has been reported in the gastric tissues of *H. pylori*-infected patients (Tatemichi et al. 1998; Fu et al. 1999; Li et al. 2001), independently of the *cagA* status of the bacteria (Son et al. 2001). Nonetheless, *NOS2* expression is directly related to the presence of the bacteria because *NOS2* is less expressed in *H. pylori*-negative gastritis than in infected patients (Fu et al. 1999; Goto et al. 1999), and in the gastric mucosa after the eradication of *H. pylori* (Mannick et al. 1996; Hahm et al. 1997; Antos et al. 2001; Felley et al. 2002).

In *H. pylori* gastritis, *NOS2* protein has been localized to the epithelium, endothelium, and lamina propria of the stomach (Fu et al. 1999; Sakaguchi et al. 1999) or suggested to be only in inflammatory cells, including PMNs and mononuclear cells (Mannick et al. 1996; Goto et al. 1999; Sakaguchi et al. 1999; Felley et al. 2002).

Similarly, increased *Nos2* mRNA and protein has been observed in isolated gastric macrophages of *H. pylori* SS1-infected C57BL/6 mice after 4 months (Touati et al. 2003; Chaturvedi et al. 2010; Lewis et al. 2011). Multiple regulators of macrophage *NOS2* expression during *H. pylori* infection have been elucidated. Deletion of arginase 2 (Lewis et al. 2011; Hardbower et al. 2016a) and inhibition of ODC (Chaturvedi et al. 2010) or heme oxygenase-1 (Gobert et al. 2014) results in increased *NOS2* levels and NO production in vitro and in gastric macrophages. Further, we recently reported that EGFR is phosphorylated in macrophages in response to

H. pylori in vitro and in gastric macrophages of mice and humans, and this is essential for M1-type macrophage activation and NOS2 expression (Hardbower et al. 2016b). The expression of *Nos2* mRNA has also been evidenced in Mongolian gerbils infected with *H. pylori* for 2 weeks (Matsubara et al. 2004) or 3 months (Elfvin et al. 2006). Lastly, like in humans, a reduction of *Nos2* mRNA is observed with the eradication of *H. pylori* in infected INS-GAS mice (Lee et al. 2008).

Interestingly, nitrotyrosine, which indicates the nitration of tyrosine by peroxynitrite (ONOO^-) generated from NO and O_2^- , is immunodetected in epithelial cells and macrophages, even in studies in which NOS2 is found only in inflammatory cells (Mannick et al. 1996; Goto et al. 1999; Sakaguchi et al. 1999). This suggests that NO is effectively synthesized from NOS2 and that reactive nitrogen intermediates (RNI) target the cells surrounding NOS2-expressing macrophages, thus providing a rationale for a potential biological effect in the infected tissues.

4.2 NO-Mediated *H. pylori* Carcinogenesis

Although the level of *H. pylori* gastritis is similar in wild-type and *Nos2*^{-/-} mice (Miyazawa et al. 2003; Obonyo et al. 2003; Hardbower et al. 2016a), several reports suggest that NO may have an effect on *H. pylori*-mediated carcinogenesis. First, NO and certain RNI are considered to be potent mutagens. Hence, *H. pylori* gastritis is associated with enhancement of epithelial cell content of 8-nitroguanine (Ma et al. 2004; Katsurahara et al. 2009), one of the major products formed by the reaction of guanine with ONOO^- (Yermilov et al. 1995), which enables G:C → A:T transversions, one of the most common mutations in the p53 tumor suppressor gene in gastric carcinogenesis (Tsuji et al. 1997). Thus, increased NOS2 protein level, nitrotyrosine immunostaining, and oxidative DNA damage have been detected in patients with gastric cancer compared to individuals with *H. pylori* gastritis (Goto et al. 1999; Hirahashi et al. 2014). In addition, it has been observed that *Nos2* expression in gastric tissues parallels the gene mutation frequency in gastric epithelial cells (Touati et al. 2003). Accordingly, in mice infected with *H. pylori* SS1, DNA fragmentation is observed in wild-type mice, but not in *Nos2*^{-/-} mice, despite the same level of acute inflammation (Miyazawa et al. 2003).

4.3 NOS2 Polymorphisms

Different polymorphisms have been described in the promoter region of the *NOS2* gene. A long (CCTTT) repeat (>13) in the 5' region or the -954G/C and -1173C/T single nucleotide polymorphisms have been associated with increased mRNA expression. The long (CCTTT) repeat and the -954G/C, but not -1173C/T, have been linked with an increased risk of gastric cancer in a Japanese (Tatemichi et al.

2005; Kaise et al. 2007; Sawa et al. 2008) and Brazilian population (Jorge et al. 2010), respectively, providing a rationale for the involvement of NOS2-derived NO in *H. pylori*-associated carcinogenesis.

5 The Host and Bacteria Coevolution: The Crystal Ball of *H. pylori* Pathogenesis?

Hundreds of thousands of years of coevolution have likely shaped the crosstalk between *H. pylori* and the human innate response. Several localized regions of the world with separate outcomes of *H. pylori* infection may help in the understanding of how these interactions may affect bacterial pathogenesis.

Amerindians living in the Peruvian Amazon and infected with *H. pylori* exhibited active gastritis and intestinal metaplasia, but none had peptic ulcer or gastric cancer (Suzuki et al. 2011). The *H. pylori* isolates harbored by these individuals belong to Amerindian strains (hspAmerind); however, the authors have highlighted that these strains possess two types of CagA proteins, namely Amerindian-I (AM-I) and Amerindian-II (AM-II), with particular features (Suzuki et al. 2011). First, AM-I and AM-II have altered Glu-Pro-Ile-Tyr-Ala (EPIYA)-B motifs, which are ESIYT and GSIYD, respectively. Second, the CRPIA, which is responsible for the phosphorylation-independent signaling of CagA, is different from those of Western or East Asian strains; further, AM-I CagA shows two AM-I CRPIA motifs, whereas AM-II CagA have either one AM-II motif or an AM-I plus an AM-II motif. Third, the N-terminal region of the AM-II CagA, involved in plasma membrane anchoring, lacks two large internal segments. The authors next questioned whether these particular CagA motifs could be associated with lower gastric cancer risk. The level of CagA phosphorylation in gastric epithelial cells associated with these particular Amerindian strains does not differ from that associated with Western or East Asian CagA, suggesting that the modified EPIYA motifs in AM-I and AM-II CagA do not have a functional effect (Suzuki et al. 2011). Nonetheless, the authors demonstrated that CXCL8 and cancer-associated Mucin 2 were produced in lower amounts by cultured epithelial cells or the gastric mucosa of gerbils during infection with *H. pylori* with AM-I or AM-II CagA compared to strains harboring Western or East Asian CagA (Suzuki et al. 2011). This effect was attributed to the modified CRPIA motifs that caused decreased interactions between CagA and MET proto-oncogene, receptor tyrosine kinase (MET, c-Met), and less epithelial cell responses to *H. pylori* (Suzuki et al. 2011). The discovery that this particular AM CagA of the native Amerindian plays a major role in the attenuation of the inflammation and consequently to less carcinogenesis is reinforced by the fact that other native Peruvians with high risk to develop gastric cancer are infected with *H. pylori* that possess a Western-type *cag* pathogenicity island (Devi et al. 2006).

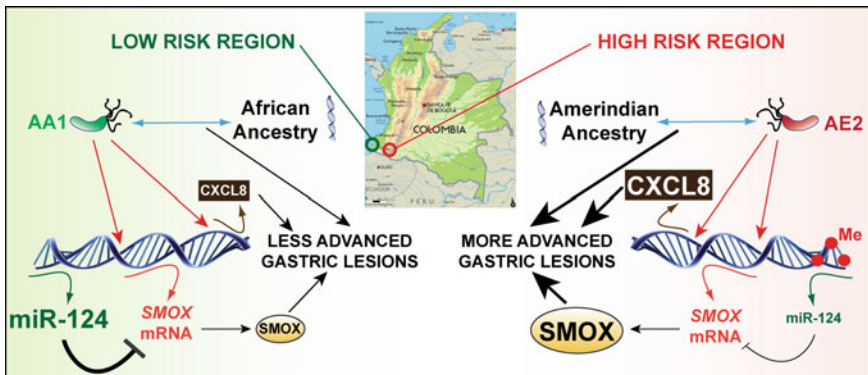


Fig. 3 A hypothesis to resolve the so-called Colombian enigma. The African strains (AA1 phylogenetic group) belonging to the low-risk (LR) region induce less CXCL8 than European strains (AE2) of the high-risk (HR) region. The methylation (Me, methyl) of the *miR-124* gene is greater in gastric tissues from the HR versus LR region; therefore, miR-124 is less expressed and SMOX protein more translated in subjects from the HR region. Moreover, individuals with an African ancestry infected with AA1 isolates have less risk for developing more advanced gastric lesions than Amerindians infected with AE2 *H. pylori* strains

In Colombia, the incidence of gastric cancer is 25 times higher in the towns of Tuquerres and Pasto, in the Andes Mountains [high-risk (HR) region] than in the town of Tumaco located on the coast [low-risk (LR) region] (Correa et al. 1976). HR *H. pylori* isolates were found to all be of European phylogeographic origin, whereas one third of the LR strains were of European origin, and two-thirds had an African origin (de Sablet et al. 2011). Furthermore in the cases from the LR region, the strains associated with higher levels of gastric epithelial DNA damage and premalignant lesions in the source patients were of European phylogeographic origin (de Sablet et al. 2011). Gastric tissues from subjects from the HR region exhibit greater levels of SMOX protein and oxidative DNA damage than those from LR region (Chaturvedi et al. 2015). Also, the HR European isolates induced more CXCL8 and SMOX expression in AGS cells than the clinical strains with an African phylogeographic origin (Sheh et al. 2013; Chaturvedi et al. 2015) (Fig. 3). Although European *H. pylori* express more *cagA*, *vacA*, and *babB* (Sheh et al. 2013) than LR strains, the differences in the induction of gene expression in AGS cells by HR and LR isolates were not attributable to differences in CagA expression and phosphorylation (Chaturvedi et al. 2015). Inversely, the African strains, but not the European ones, significantly induced apoptosis in gastric epithelial cells (Sheh et al. 2013; Chaturvedi et al. 2015).

Furthermore, the hypermethylation, and thus the silencing, of the genes encoding miR-124, the microRNA regulating SMOX expression (Murray-Stewart et al. 2016), has been observed in patients with *H. pylori*-associated gastric cancer (Ando et al. 2009). Increased DNA methylation of three *mir-124* gene loci has recently been evidenced in the HR Colombian Andean subjects compared to the LR coastal inhabitants (Murray-Stewart et al. 2016), demonstrating that the epigenetic control

of this miRNA, which is known as a tumor suppressor, could also have an important effect on the innate response of epithelial cells and consequently on *H. pylori*-mediated carcinogenesis (Fig. 3). It would be of interest to determine whether *miR-124* methylation is only associated with the type of *H. pylori* infection or is also dependent on the ethnic origin and genetic features of the human population.

Studies from our group have described that both the host and *H. pylori* phylogeographic variations, rather than the phylogenetic categorization of the bacteria per se, are essential for the development of gastric disease progression along the Correa cascade (Kodaman et al. 2014). In the LR region, humans exhibit a predominant African ancestral cluster and have less chance to develop severe gastric lesions, because they are mainly infected with African *H. pylori* strains, indicative of ancestral coevolution of the bacterium and the host (Fig. 3). In contrast, the risk of more advanced lesions in the histologic cascade toward cancer is increased in individuals showing a predominant Amerindian ancestry, but infected with strains exhibiting a substantial African component (Kodaman et al. 2014). In the HR region, the mountain population is principally Amerindian, but are infected mainly with *H. pylori* strains belonging to a predominant European origin suggesting a loss of coevolution. Further, in the HR mountain region, strains with a significant African component (~20%) were particularly associated with the development of more severe gastric lesions, especially intestinal metaplasia and dysplasia (Kodaman et al. 2014) (Fig. 3). This host/pathogen crosstalk has been found to be more predictable for the risk of *H. pylori*-mediated diseases than the effect of *cagA* (Kodaman et al. 2014).

Therefore, human and bacterial phylogenetic parameters should be taken into consideration for an accurate determination of the effect of the host response in the development of gastric diseases associated with *H. pylori* infection. More studies in additional human populations are needed to further evaluate the interactions of the bacterium and the host that predict disease outcome.

6 Concluding Remarks

The host innate immune response is certainly one of the major events involved in *H. pylori* pathogenesis. We focused our review on CXCL8, ROS, and NO, since these effectors have been shown to exhibit function in infectious inflammation and carcinogenesis. However, numerous other products of the innate response, including cytokines and other chemokines, prostaglandins, and their metabolites, may be involved in the etiology of *H. pylori*-related diseases. While several correlations suggest that CXCL8 has an important role in the development of gastric malignant lesions, more studies are warranted to prove that it is a key oncogenic molecule during *H. pylori* infection. In contrast, the reduction of carcinoma in *H. pylori*-infected gerbils treated with inhibitors of SMOX or the upstream enzyme, ODC, provide a strong rationale for envisioning the ODC-SMOX pathway as a potential

target to limit the development of the more advanced gastric lesions. Because NOS2 has been linked to *H. pylori* carcinogenesis and NO reacts with ROS to form RNI, the study of their synergistic effect may also be a promising area of investigation. But importantly, the evidence that the phylogenetic features of the human population and/or *H. pylori* are essential for host/pathogen interaction and the outcome of the infection demonstrates the necessity to bring human ancestry into the analysis of innate responses. Our ongoing studies investigating the human and *H. pylori* genetics, utilizing whole genome sequencing in cases from LR and HR in Latin America, are expected to provide new insights into gastric cancer development.

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